STUDIES ON RAPIDLY FROZEN SUSPENSIONS OF YEAST CELLS BY DIFFERENTIAL THERMAL ANALYSIS AND CONDUCTOMETRY

PETER MAZUR
From the Biology Division, Oak Ridge National Laboratory, Oak Ridge

ABSTRACT Few, if any, yeast cells survived rapid cooling to −196°C and subsequent slow warming. After rapid freezing, the suspensions absorbed latent heat of fusion between −15° and 0°C during warming, and the relation between the amount of heat absorbed and the concentration of cells was the same as that in equivalent KCl solutions, indicating that frozen suspensions behave thermally like frozen solutions. The amount of heat absorbed was such that more than 80 per cent of the intracellular solution had to be frozen. The conductometric behavior of frozen suspensions showed that cell solutes were still inside the cells and surrounded by an intact cell membrane at the time heat was being absorbed. Two models are consistent with these findings. The first assumes that intracellular freezing has taken place; the second that all freezable water has left the cells and frozen externally. The latter model is ruled out because rapidly cooled cells do not shrink by an amount equal to the volume of water that would have to be withdrawn to prevent internal freezing.

INTRODUCTION

The presence of liquid water and a restricted range of temperatures are mandatory for active living systems. The removal of water by actual withdrawal or by conversion to ice, or the lowering of temperature lead either to death or to a reversible inactive state. Although the latter is the goal of cryobiologists interested in the preservation of living cells, one approach to this goal is to determine the causes of low temperature death. Currently two theories are in vogue. One relates cellular damage to the concentrating of solutes as ice separates out during the progressive freezing of an aqueous solution (Smith, 1961). The other suggests that injury is associated with the formation of the ice crystals themselves, and specifically of intracellular ice crystals. In both cases, the evidence is primarily circumstantial, consisting chiefly of observations on the relation between survival and such variables as the nature of the suspending medium, the temperature, the cooling and warming rates, and the lengths of exposure to subzero temperatures.

A definitive answer to the question of whether crystallization occurs in cells that are injured by low temperature exposure is essential to understanding the causes of injury; but it is also a question with broader implications, for whether intracellular freezing can occur depends partly on the rate at which internal water is able to pass through the cell membranes and partly on the ease with which it can undergo crystallization within the cell. The question thus involves cell permeability and the state and structure of cellular water at normal temperatures.

In previous studies, I have postulated that internal ice is responsible for the death of yeast and certain other microorganisms (Mazur, 1960, 1961*a, b*). Underlying this hypothesis has been the tacit assumption that the physical behavior of cells at subzero temperatures obeys the same physical-chemical laws as macroscopic dilute solutions, and is not significantly influenced by biological processes such as the active transport of water or solutes.

Let us consider suspensions of yeast cells in pure water at temperatures above 0°C to be compartments of aqueous solution separated from the external medium by membranes permeable to water but impermeable to intracellular solutes. If such suspensions are subject to the same laws as dilute macroscopic solutions at subzero temperatures, the scheme suggested in Fig. 1 is then valid: When the suspension is cooled below 0°C, ice eventually appears in the external medium. As the temperature falls below the freezing point of the protoplasm, the vapor pressure of the intracellular water becomes greater than that outside the cell. In other words, the water in the cell becomes supercooled and thermodynamically unstable relative to the external ice. Four phenomena can occur in such a supercooled cell (Fig. 1).

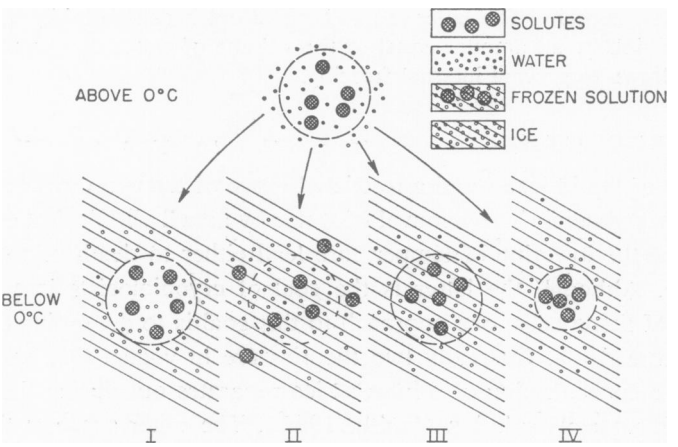

FIGURE 1 Alternative states of a cell at (A) above 0°C and (B) below 0°C with the external water frozen.

1. It can remain supercooled indefinitely if the cell contains activation energy barriers to prevent equilibrium from being attained at measurable rates (I).

2. The semipermeable plasma membrane can be destroyed (II). If it were, there would cease to be an "inside" and "outside" of the cell, and solutes would diffuse into the surrounding medium, converting the suspension into a non-compartmented aqueous solution and eliminating vapor pressure differentials.

3. The cell can undergo intracellular freezing (III). This would equalize the internal and external vapor pressures when both regions were at the same temperature. A corollary to this possibility is for extracellular and intracellular freezing to occur simultaneously so that the cell never is supercooled.

4. Intracellular supercooled water, because of its higher vapor pressure, can flow out of the cell and freeze externally (IV). This outflow would lower the vapor pressure of the protoplasm by concentrating the solutes and could continue until the cell was dehydrated rather than frozen.

It can be seen that models II and III (destroyed membranes and intracellular freezing) convert suspensions into true frozen solutions; that is, two phase systems in which solutes are in direct contact with pure ice. This will not be true of model I where the water has remained supercooled inside the cell.

One distinction between a frozen solution and pure ice, and hence between model I and models II and III is the temperature range over which melting occurs. Ice melts at 0°C, but a frozen solution begins to melt below zero at its eutectic point and melts progressively as the temperature rises. Since the fusion of ice requires 80 cal/gm, progressive melting of a frozen solution would be accompanied by the progressive absorption of heat in excess of that required to raise the temperature of a sample of pure ice of equal heat capacity. Accordingly, if samples of ice and frozen solution are given equal quantities of heat, the excess heat absorbed by the solution will cause its temperature to lag below that of the ice. The lag can be quantified most accurately and conveniently by the technique of differential thermal analysis (DTA) (Smothers and Chiang, 1958).

If frozen cells behave like frozen solutions, not only should they absorb heat during warming, but the quantity of heat should be related to the temperature and to the concentration of solutes in contact with ice. One purpose of the present paper will be to derive these relations, to describe the apparatus and procedures used in making differential thermal analyses, and to calibrate the apparatus in terms of the thermal behavior of solutions of potassium chloride, a well studied solute. We will then compare the thermal behavior of suspensions of the yeast *Saccharomyces cerevisiae* with that of KCl. These comparisons, along with information on the electrical resistance of frozen and unfrozen suspensions and on the volumes of cells at subzero temperatures, will permit a decision as to whether cells behave like frozen solutions and whether or not they contain intracellular ice.

The dehydrated cells of model IV would not be true frozen solutions, since this model, like model I, pictures the solutes as separated from external ice by the solute-impermeable membrane but they would behave thermally as though they were.

This model assumes that sufficient water leaves the cells during cooling to prevent intracellular freezing. Such dehydration would lower the internal vapor pressure to that of the external ice. During warming, the vapor pressure of ice always rises more rapidly with rising temperature than does that of a liquid solution of fixed concentration (Glasstone, 1946), and this differential would produce diffusion of water or vapor from ice outside the cell back into the solution inside; the transfer of water would be thermodynamically equivalent to melting and would result in the absorption of 80 cal of heat for each gram of water transferred.

Determining the physical state of yeast cells at subzero temperatures will, accordingly, require answers to the following questions:

1. Are cells compartments of aqueous solution at normal temperatures? The answer on the basis of conductivity measurements and differential thermal analysis is affirmative.

2. Do cell suspensions at subzero temperatures behave like frozen macroscopic solutions? The answer is again affirmative and is provided by differential thermal analysis (DTA).

3. Do the cells themselves behave like frozen solutions? The differential thermal data indicate that they do and that more than 80 per cent of the cell water is frozen. Model I is thereby eliminated.

4. Are the cell membranes disrupted during cooling (model II)? Apparently not, on the basis of electrical resistivity measurements on frozen suspensions.

5. Have the cells undergone intracellular freezing (model III) or has the water left them and frozen externally (model IV)? Since suspensions will behave like frozen solutions on the basis of either model, they cannot be distinguished by DTA. However, as depicted in Fig. 1, their behavior can be differentiated by changes in cell volume.

METHODS AND MATERIALS

KCl Solutions. KCl solutions were prepared as molar solutions with analytical grade crystals and deionized glass-distilled water. In the range of concentrations used (0.001 to 0.1M), molarity and molality are equivalent to within 0.5 per cent or less (*e.g.*, 0.1000 molar KCl = 0.1004 molal KCl); and therefore the two terms for concentration will be used interchangeably without correction.

Cell Culture and Preparation of Suspensions. Subcultures of *Saccharomyces cerevisiae*, diploid strain NRRL-Y-2235, were prepared as described elsewhere (Mazur, 1961*a*) by incubating for 24 hours at 29°C in liquid medium in Fernbach flasks on a shaker. The resulting 400 to 500 ml of suspension was washed 7 times in deionized distilled water, concentrated to 8 ml, transferred to a Kolmer centrifuge tube and washed 3 more times in water. The final supernatant was then decanted, and sufficient deionized water added to make the volume of the packed cells 45 per cent of the suspension, the volume of which was 1.8 to 2.0 ml. This concentration of cells will be denoted 1×. Suspensions of one-half and one-quarter this concentration ($\frac{1}{2}\times$ and $\frac{1}{4}\times$) were prepared by adding sufficient water to packed cells to yield the desired concentration, or by diluting

a previously prepared 1 × suspension. Centrifugations were at 15°C at 500*g* for 7 minutes.

Differential Thermal Apparatus. The apparatus in Fig. 2 is a modification of those described by Skau (1933) and Rey (1960). Four Lusteroid centrifuge tubes (Lourdes Instrument Corp., Brooklyn, New York; 10 × 80 mm) were held symmetrically in a Lucite circular lid. An opposing pair of tubes received 1.50 ml of either KCl

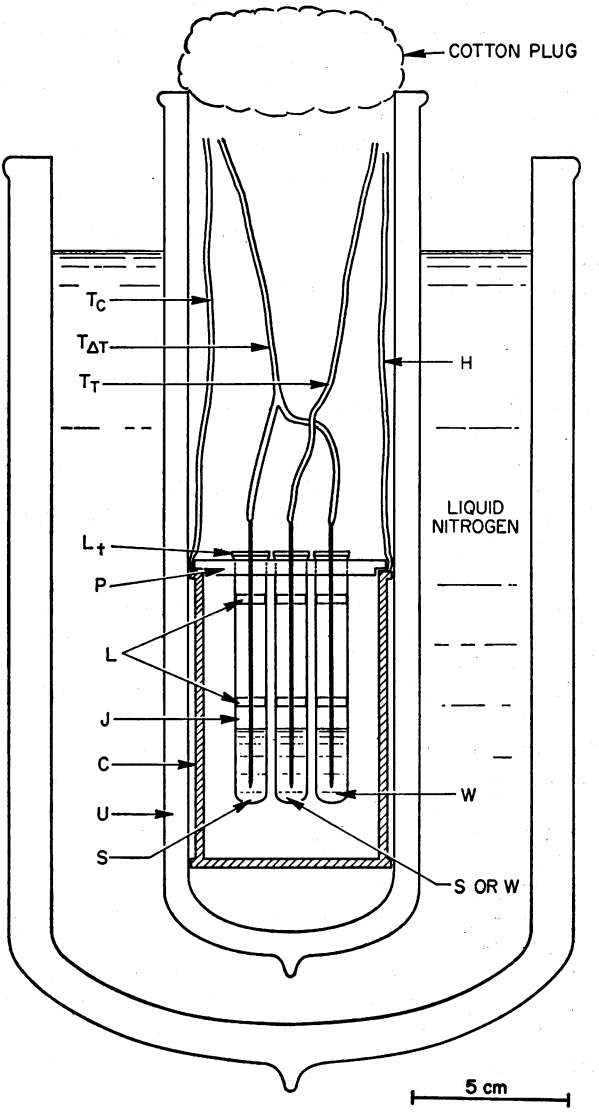

FIGURE 2 Apparatus for differential thermal analysis. T_c, cylinder thermocouple; $T_{\Delta T}$, differential thermocouple; T_T, absolute thermocouple; L_t, large Lucite washers; *P*, Lucite lid; *L*, Lucite washers; *J*, Lusteroid tube; *C*, brass cylinder; *U*, unevacuated, unsilvered Dewar flask; *S*, solution or suspension; *W*, water or ice; *H*, leads from Nichrome wire to Variac. A fourth Lusteroid tube is behind the center one.

solution or cell suspension and 1.50 ml of deionized distilled water, respectively. Into each tube was also inserted the junction of a differential thermocouple to measure the difference in temperature between the solution or suspensions and the water. A third tube received 1.50 ml of water or the test material along with the measuring junction of a second thermocouple which had its reference junction in an ice-water mixture at 0°C in a Dewar flask. This thermocouple indicated the actual temperature of the sample. The fourth tube opposite the third was a dummy containing 1.5 ml of water and a thermocouple assembly, and merely served to complete the symmetry. The thermocouples were 40 gauge copper-constantan wire (Thermo-Electric Co., Inc., Saddle Brook, New Jersey) cemented in stainless steel tubes cut from 16 gauge hypodermic needles and the same couples were used in all experiments. Lucite washers were cemented to the needle with Permount (Fisher Scientific Company, New York) and were machined to fit the Lusteroid tubes with little tolerance so that they centered the thermocouples in the tubes. Larger washers held the thermal junctions immersed to the same depth in the samples in each experiment.

Voltages from the differential and absolute thermocouples were fed to separate DCμv amplifiers (model 9835, Leeds and Northrup Company, Philadelphia). The amplifier outputs drove separate self-balancing recording 10 mv potentiometers (Leeds and Northrup Speedomax H and Brown Electronik, Minneapolis-Honeywell Regulator Co., Philadelphia) or an XY recorder (model 135, F. L. Moseley Co., Pasadena).

The Lucite lid holding the sample tubes sat on a brass cylinder, the circumference of which was wrapped with about 6 meters of 30 gauge Nichrome wire (Driver-Harris Co., Harrison, New Jersey) with a resistance of 120 ohms. The heat produced by the Nichrome was controlled by a Variac auto transformer (General Radio Company, West Concord, Massachusetts) powered by a Sola constant voltage transformer (Sola Electric Co., Chicago).

The brass cylinder, in turn, was firmly positioned by its top and bottom flanges in an unevacuated and unsilvered Dewar-type flask. The flask was immersed to 1 to 2 inches of its top in a 4 liter Dewar of liquid nitrogen. The temperature of the cylinder was recorded by a fourth thermal junction soldered to its flange, the output going to a Speedomax G recording potentiometer.

Procedure in Mdking Differential Thermal Analyses. The Lusteroid sample tubes with thermocouples in place were positioned in the Lucite lid and two-thirds immersed in a separate bath of liquid nitrogen for 45 seconds. The sample temperatures fell below −100°C in 20 to 30 seconds. The lid was then quickly transferred to the brass cylinder which had been previously equilibrated at −30° to −35°C by supplying current to the heating coil. The test solution or suspension equilibrated at −30° to −35°C in about 15 minutes, whereupon warming was initiated by increasing the voltage to the Nichrome heating wire from 46 to 63 ±1 volts. The latter voltage was maintained until all samples had thawed, and yielded a warming rate for the pure ice of 1.32 ±0.02°C/minute between −30° and 0°C. Thawed suspensions were often subjected to a second cycle of freezing and thawing. The procedure was the same.

Calculations and Computations. Thermocouple temperatures were recorded in microvolts. The differential temperatures were read to ±0.5 μv and were accurate to ±1 μv. The absolute temperature was read to ±2 μv and was accurate to ±5 μv. These EMF's were converted to degrees Celsius by calibration between −30° and 0°C against a mercury-in-glass thermometer certified by the National Bureau of Standards. The conversion factors were close to the published values for copper-constantan thermocouples (36 to 39 μv/°C, depending on the temperature).

Conductivity Measurements

A. *Liquids.* Conductivities of yeast suspensions and supernatant fluids were measured with microcells constructed of two rectangular plates of 34-gauge platinum, about 8 × 12 mm, held 1 to 2 mm apart by glass or epoxy spacers, and platinized. The cells fitted inside the Lusteroid centrifuge tubes. Their cell constants were close to 0.100, and were determined in KCl solutions ranging in concentration from 0.005 to 0.1M. During conductivity readings, the samples were immersed in a water bath at 23° to 27°C. The temperature was measured to 0.1°C and the conductivity readings corrected to 25°C using a temperature coefficient of 2 per cent per degree (Robinson and Stokes, 1959). The Wheatstone bridge was an Industrial Instruments model RC-1 (Industrial Instruments, Inc., Cedar Grove, New Jersey) operating at 60 CPS and occasionally the apparatus described below.

B. *Frozen Suspensions and Solutions.* Resistivities of frozen cell suspensions and solutions of KCl were measured during warming with these microconductivity cells. The measuring junction of a 40-gauge copper constantan thermocouple was fastened to the outside of one of the two electrodes or between them. The conductivity cell was then inserted into a Lusteroid centrifuge tube containing yeast suspension or KCl solution; the sample was frozen to −196°C, equilibrated at around −35°C, and warmed and thawed in the differential thermal apparatus using the procedures described. Thermocouple outputs were measured potentiometrically.

Resistances of the sample were measured with a General Radio model 650 A impedance bridge. The measured resistances of frozen samples were as high as 10^8 ohms, and to balance the bridge it was necessary to shunt one of the ratio arms with a Leeds and Northrup model 4775 DC resistance box. A General Radio model 1210 C RC oscillator was used as the generator and a Model 1232 A AC amplifier as the detector. The latter was sensitive to unbalances of less than 1 μv. The resistors in the DC resistance box were nearly constant over the frequencies used and caused no problem. Reactances were balanced by decade capacitors placed across the appropriate arm, and all external wiring was shielded. The bridge was checked for proper functioning against known wire-wound and carbon megohm resistors. It yielded sharp nulls with both resistors and frozen material. During the warming of the latter, the bridge was accurately nulled for resistance readings every ½ to 2 minutes, but kept approximately nulled throughout a run to avoid heating of the sample or the bridge resistors.

Viable and Total Cell Counts and Estimates of Cell Volume. The percentages of cells surviving low temperature exposure were estimated by a dilution plate count procedure. Total counts were made on hemocytometer slides after appropriate dilutions of the suspension. A hematocrit technique was used to measure the volumes of packed cells. It involved filling precision-bore capillaries (0.6 mm I.D ±0.01 mm, Drummond Scientific Co., Philadelphia) with suspensions, spinning them at 15,500 *g* for 30 minutes in a Clay-Adams microhematocrit centrifuge, and measuring the relative cell pack volumes on a Drummond hematocrit reader. Cell volumes were also estimated from micrometric measurements of cell diameters. Details of these procedures have been reported previously (Mazur, 1961*a*, *b*).

Water Contents and the Density and Heats of Solution of Yeast Solids. The amount of water in suspensions was estimated by drying them to constant weight in an oven at 104 ±2°C. The density of the cell solids was measured in Gay-Lussac–type pycnometers. The heats of solution of dried cell solids were calculated by measuring the temperature change produced by adding the solids to 100 gm of water in a calorimeter,

the apparatus being that described by Daniels *et al.* (1956) except that heating wires were eliminated and temperatures were measured with a thermocouple. The measurements of density and heats of solution were made mostly with commercial active dry yeast (Fleishmann's), both without further treatment and after oven drying; but some were made with freeze-dried NRRL-Y-2235 cells.

Sonication. A DTA Lusteroid tube containing 1.5 ml of 1× suspension was placed in 60 ml of water in the cavity of a Raytheon (Waltham, Mass.) 10 kc sonic distintegrator for 2 hours at 10°C.

Statistical Analyses. Where appropriate, data were subjected to regression or other statistical analyses. Statements of significance are based on the 0.05 level of probability unless stated otherwise. The symbols $s_{\bar{x}}$, $s_{y.x}$, s_{λ}, n and P refer to the standard error of the mean, standard error of a regression curve, standard error of the slope, number of observations, and probability, respectively. Regressions were weighted according to the number of replicates for a given concentration. Regression statistics are given in the figure legends. Standard errors of the mean are indicated by vertical brackets in the figures and are enumerated in Appendix B, Table IX.

CALIBRATION WITH KCl SOLUTIONS

Warming Curves of Pure Ice and KCl Solutions. The thermal behavior during warming of 1.5 gm of frozen 0.05 M *KCl* solution is compared with that of 1.5 gm of ice in Fig. 3. The observed curves approximated the ideal (Glasstone,

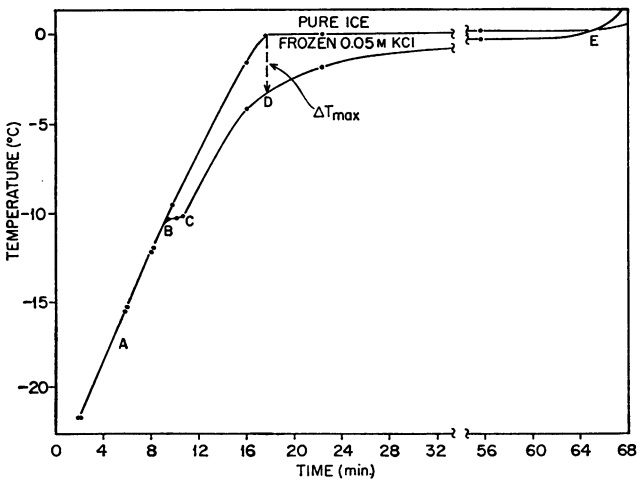

FIGURE 3 Warming curves of frozen 0.05 M KCl and of ice. The points merely serve to distinguish the two curves which were redrawn from continuous recorder tracings. The several lettered regions are discussed in the text.

1946). The temperature of the pure ice rose nearly linearly with time to 0°C, and remained at 0°C until all the ice had melted. The temperature of the frozen solution also rose nearly linearly with time in region *A*, but then, as expected, diverged sharply at *B* (−11°C) which is the eutectic point of KCl solutions (actually

−10.7°C, Seidell, 1940). From C to E, the solution temperature rose at a diminishing rate as part of the incoming heat was absorbed in progressive melting of ice and part in raising the temperature of the two phase system. This absorbed heat produced a difference in temperature (ΔT) between the two samples which increased to a maximum (ΔT_{max}) the instant the ice reached 0°C and then receded. Finally at E (the melting point) all the ice melted and all further heat went to raise the temperature of the now totally liquid solution.

Since the thermal lag produced by heat absorption was small, it would be a valid measure of melting only if the samples of ice and of solution warmed at nearly identical rates when melting was not occurring (*e.g.*, in region A of Fig. 3). The differential thermal technique was better able to meet this requirement because it compared the temperatures of the samples simultaneously; moreover, it had the advantage that the quantity of interest, ΔT, was measured directly and was less subject to instrumental and thermocouple errors than if it had been obtained by subtraction from separate warming curves for the solution and for the ice. Since ΔT is the pertinent quantity, it will be plotted directly instead of using absolute temperatures as in Fig. 3.

Two types of differential plots are required to present the complete information. One is a plot ΔT *versus* time (t), such as that in Fig. 4 which compares four con-

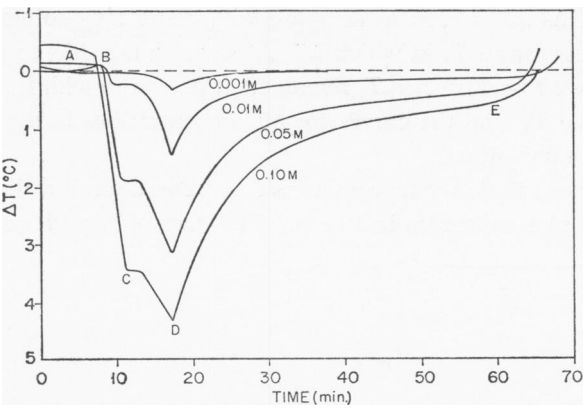

FIGURE 4 Differential thermal analysis (ΔT *versus* time) of frozen KCl solutions during warming. The reference was pure ice.

centrations of KCl *versus* ice. The curve for 0.05 M KCl represents the same data as in Fig. 3, and the various regions are indicated by the same lettering. This plot emphasizes the thermal behavior after ΔT_{max} has been reached and permits an estimate of the melting point which was taken as the temperature at which $d\Delta T/dt$ was minimum (region E).

The second type is the plot of ΔT *versus* the temperature of the solution, shown in Fig. 5. This plot emphasizes the events occurring before the attainment of ΔT_{max}

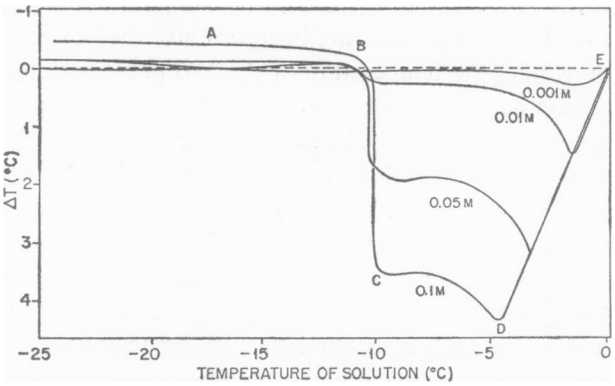

FIGURE 5 Differential thermal analysis (ΔT *versus* temperature) of frozen KCl solutions during warming. The reference was pure ice.

and shows the relation between temperature and heat absorption. The shift of ΔT_{max} to the right along the abscissa with decreasing concentrations of KCl appears peculiar, but actually is a necessary consequence of the relations between ΔT and the solution temperature T_s. By definition,[1] $\Delta T = T_s - T_i$ where T_i is the temperature of the ice. Hence, when $T_i = 0°C$, $\Delta T = T_s$. Also when T_i first reaches 0°C, $\Delta T \equiv \Delta T_{max} = T_s$. The value of ΔT_{max} is seen to decrease with decreasing solute concentrations; therefore the temperature T_s at which ΔT_{max} occurs must shift toward 0°C to preserve the equality $\Delta T = T_s$. Since T_i remained at 0°C even with further heating, ΔT continued to equal T_s, and the curves for all concentrations in Fig. 5 approach the melting point with unit slope.

The quantitative relation between ΔT_{max} and the concentration of solute was derived from the phase diagram in Fig. 6. If a frozen KCl solution of original con-

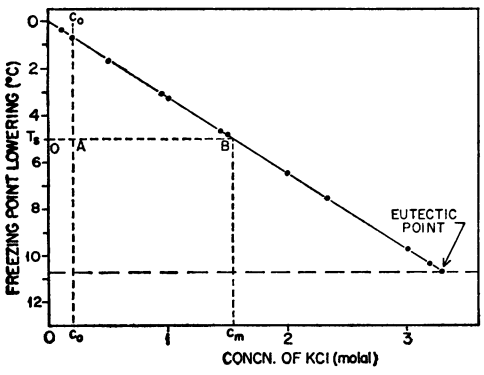

FIGURE 6 Phase diagram of the freezing point lowering *versus* the molal concentration of KCl. Derived from cryoscopic data in Seidell, 1940 and the International Critical Tables, 1928.

[1] T_s and T_i will be considered to be degrees below 0°C. This will make them and all other parameters positive and thus avoid difficulties with the logarithms of negative numbers.

centration c_o molal is warmed, the fraction q of ice melting at any temperature T_s above the eutectic will be the ratio of $\overline{OA}/\overline{OB}$ at that temperature (Glasstone, 1946). Or

$$q = \frac{\overline{OA}}{\overline{OB}} = \frac{c_o}{c_m} \tag{1}$$

where c_m is the molal concentration of solutes in the melted portion. Furthermore, when T_s is a linear function of c_m, as is nearly true for KCl, then

$$T_s = bc_m \tag{2}$$

where b is the slope of the freezing point curve, or the molal freezing point lowering.

Multiplying (1) by (2), we obtain

$$q \cdot T_s = bc_o \tag{3}$$

Since q is the fraction of ice melted, it is also the fraction of the total latent heat of fusion that has been absorbed upon warming to a given temperature (assuming the latent heat of fusion is constant with temperature, an approximation introducing an error in ΔT of about 0.6 per cent per degree below 0°C). The heat absorbed in melting will not have been available to raise the temperature of the frozen solution, which consequently will lag behind the temperature of the sample of ice by ΔT degrees. If the heat capacity of the frozen sample is C_s cal/deg, the heat absorbed by melting will produce a temperature lag (ΔT) of

$$\Delta T = \frac{q \cdot \Delta H_f}{C_s} \tag{4}$$

where ΔH_f is the total latent heat of fusion of the sample in calories.

Solving equation (4) for q and substituting in (3), we have

$$\Delta T\, T_s = c_o \frac{\Delta H_f b}{C_s} \tag{5}$$

But, by definition $T_s = \Delta T + T_i$; therefore

$$\Delta T(\Delta T + T_i) = c_o \frac{\Delta H_f b}{C_s} \tag{6}$$

When $T_i = 0°C$, $\Delta T = \Delta T_{max}$, and therefore,

$$(\Delta T_{max})^2 = c_o \frac{\Delta H_f b}{C_s} \tag{7}$$

With KCl solutions as dilute as 0.1 molal, the quantity $\Delta H_f b/C_s$ should be nearly constant. From Fig. 6, b is calculated to be 3.24 deg/molal unit. For a 1.5 gm sample, ΔH_f is 80 × 1.5 or 120 calories. On the other hand, the value of C_s rises as ice with specific heat of 0.5 cal/gm/deg is converted to water with specific heat of 1

cal/gm/deg; but it changes little in solutions no more concentrated than 0.1 molal, for less than 7 per cent of the ice melts in the interval when ΔT is increasing to ΔT_{max}, and it is this interval to which these equations apply. (That less than 7 per cent melts can be seen by calculating by means of equation (3) the fraction melted (q) at the temperature at which ΔT is maximum.) The heat capacity of the solution will thus not only be nearly constant, but since the solutions are dilute, it will nearly equal the heat capacity of the reference sample of 1.5 gm of pure ice; namely, 0.75 cal/deg.

Equation (7) in logarithmic form is

$$\log (\Delta T_{max})^2 = \log c_o + \log \frac{\Delta H_f b}{C_s} \tag{8}$$

Since the last term is nearly constant, a plot of log $(\Delta T_{max})^2$ *versus* log c_o should be linear with unit slope, which it was (Fig. 7). The DTA curves of ΔT versus time

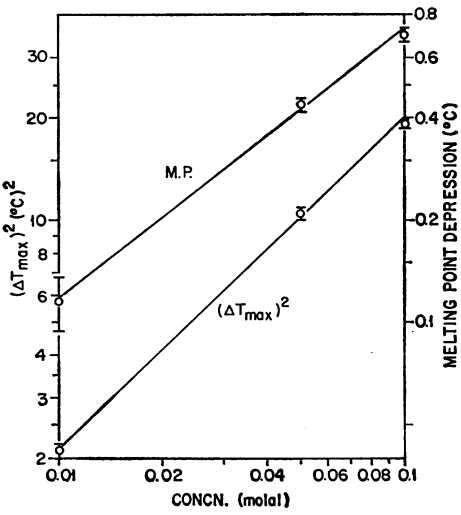

FIGURE 7 Relation between the observed values of $(\Delta T_{max})^2$ and melting point depressions and the concentration of KCl. The regression equation of the former is log $(\Delta T_{max})^2 = 2.276 + 0.976 \log c_o$, with $s_\lambda = \pm 0.030$, $s_{y.x} = 0.047$, and $n = 13$. The curve does not depart significantly from linear nor its slope from 1 ($P > 0.5$). That of the latter is log $T_m = 0.666 + 0.798 \log c_o$ with $s_\lambda = \pm 0.083$, $s_{y.x} = \pm 0.131$, and $n = 15$. The curve does not depart significantly from linear ($P > 0.5$) but its slope is significantly less than 1 ($P < 0.05$).

also yielded estimates of melting point depressions. A plot of the logarithm of these as a function of the logarithm of KCl concentration is shown in Fig. 7 and it too is linear. Although reproducibly related to solute concentration, the absolute values of both $(\Delta T_{max})^2$ and melting point depression differed from theoretical. With the former, for example, the observed value of $\Delta H_f b/C_s$ was 189 whereas the theoretical value is 518 deg^2/molal unit. The discrepancies probably arose from assuming equilibrium conditions in deriving equation (8) when in fact equilibrium is not present in DTA. However, because the purpose of using KCl was to calibrate the apparatus in terms of a solution, the thermal properties of which are known accurately by other methods, the discrepancies are not serious. What is important is (1) that the observed temperature differentials were due to the absorption of latent

heat of fusion and (2) that the magnitudes of the maximum temperature difference and the melting point depressions were experimentally reproducible functions of solute concentration.

RESULTS

Thermal Behavior of Frozen Cell Suspensions. If yeast cells behave like frozen solutions, their thermal response during warming should be described by equations analogous to those applicable to KCl. For a system consisting only of frozen cells with no external ice, equations (5) and (7) would apply directly. But suppose the same volume is converted to a suspension with a fraction, a_w, of the total mass of water inside the cells and $(1 - a_w)$ outside. When the suspension is frozen and then warmed, only the fraction a_w that froze internally and was in contact with solutes would undergo melting at temperatures below 0°C and, therefore, only the latent heat of this fraction would contribute to the thermal lag, ΔT. Since the other quantities in equations (5) and (7) are not affected by the compartmentation, these equations become

$$\Delta T\, T_s = a_w \frac{\Delta H_f b c_o}{C_s} = a_w m \tag{9}$$

when m is approximately constant provided b is constant. And

$$(\Delta T_{max})^2 = a_w \frac{\Delta H_f b c_o}{C_s} = a_w m \tag{10}$$

If some of the internal water left the cells and some froze internally, these equations are still applicable. The loss of water would reduce the fraction a_w to, say, a_w/k, but concomitantly it would increase the internal solute concentration to $k \cdot c_o$.

Equations (9) and (10) also apply if cooling destroys the compartmentation as in model II of Fig. 1. Then n_2 moles of solute in a_w kilograms of water in intact cells will be distributed in 1 kg of water, changing the concentration from c_o molal to $n_2/1 = a_w \cdot (n_2/a_w) = a_w c_o$ molal. Simultaneously, the fraction of total water (or ice) in contact with solutes will change from a_w to 1 (the total water of the suspension), and equations (9) and (10) will remain unchanged.

Water and Solids Contents of Cell Suspensions. The equations relating heat absorption to the melting of frozen solutions require that the heat capacities of the solution and of the reference ice sample be equal, and also require knowledge of the mass fraction of the water that is sequestered in the cells. These data are listed in Table I, and the procedures used to obtain them are given in Appendix A. The heat capacity of the 1.5 gm of ice in the reference sample was 0.75 cal/deg; that of the three concentrations of frozen cell suspensions was almost identical, the maximum difference being only 1.7 per cent.

Survival. Fewer than 1 cell out of 30 million survived rapid cooling to −196°C and subsequent slow warming.

TABLE I

CALCULATED HEAT CAPACITIES OF YEAST SUSPENSIONS AND FRACTIONS OF SUSPENSIONS OCCUPIED BY CELLS AND WATER

See Appendix A for details.

Concentration of cells	a_v*	a_w‡	Heat capacity	a_v^{rel}	a_w^{rel}	Relative heat capacity§
			cal/°C			
1 ×	0.350	0.286	0.737	1	1	0.983
½ ×	0.175	0.138	0.745	0.5	0.484	0.993
¼ ×	0.0875	0.068	0.747	0.25	0.237	0.996
0 (ice)	—	—	0.750	—	—	1

* Ml cells/ml of suspension.

‡ Gm water in protoplast/gm water in suspension.

§ Relative to 1.5 gm of pure ice.

Warming Curves for Frozen Cell Suspensions. Differential thermal plots for 1×, ½×, and ¼× cell suspensions *versus* pure ice are shown in Figs. 8 and 9. In Fig. 8, the differences in temperature (ΔT) are plotted against time. In Fig. 9, the

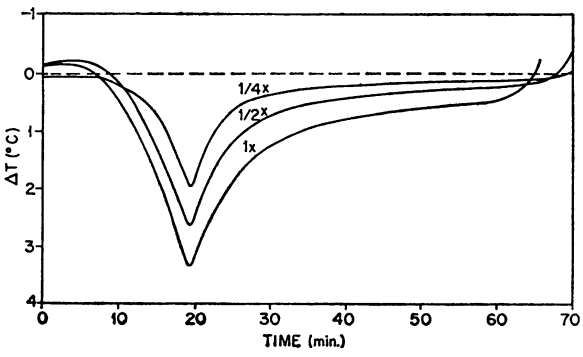

FIGURE 8 Differential thermal analyses of three concentrations of frozen yeast suspensions (ΔT *versus* time). The reference was pure ice.

ΔT's of comparable suspensions are plotted against the temperature of the suspension (T_s). The suspensions began to absorb heat above −15°C, and with further rise in the temperature the heat absorption produced an increasing difference in temperature between the suspensions and pure ice, up to a maximum (ΔT_{max}). Increasing the concentration of cells increased the magnitude of ΔT_{max} and also shifted the temperature at which it occurred. The reasons for this shift were discussed with Fig. 5.

Evidence that Frozen Cell Suspensions Behave Thermally Like Frozen Solutions. A number of facts show that the observed endothermic process was the melting of ice and that melting occurred in a manner characteristic of a frozen solution.

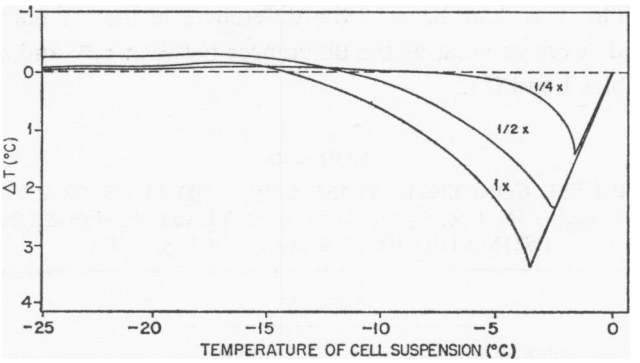

FIGURE 9 Differential thermal analyses of three concentrations of frozen yeast suspensions [ΔT *versus* the temperature of the suspensions (T_s)]. The reference was pure ice.

1. Equation (10) predicts that a plot of log $(\Delta T_{max})^2$ *versus* the logarithm of the fraction of water in the cells (log a_w or log a_w^{rel}) will be linear with unit slope if the cell suspension is a frozen solution with a constant molal freezing point lowering, b. (Log-log plots are used because of the simplicity of testing for unit slope and because our concern will be with the ratios of the values.) This plot is shown in Fig. 10, where it is compared with that for KCl solutions. The curve for the cell suspen-

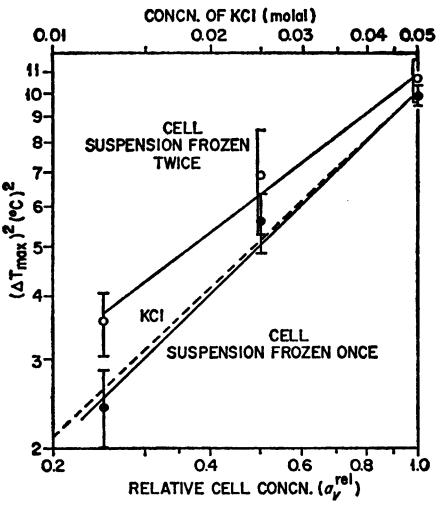

FIGURE 10 Heat absorbed during the warming of frozen yeast suspensions as a function of the relative concentration of cells compared to that absorbed by KCl solutions as a function of solute concentration. The plot for initially frozen suspensions follows the equation log $(\Delta T_{max})^2 = 1.006 + 0.999 \log a_v^{rel}$, with $s_{y.x}$ ± 0.097, $s_\lambda = \pm 0.095$, and $n = 17$. It does not depart significantly from linear ($P > 0.20$), and its slope does not depart significantly from 1 ($P > 0.5$) nor from that of KCl ($P > 0.8$). That for twice-frozen suspensions is log $(\Delta T_{max})^2 = 1.038 + 0.778 \log a_v^{rel}$ with $s_{y.x}$, s_λ, and n being ± 0.096, ± 0.136, and 8. It too is linear ($P > 0.5$) and its slope not significantly different from 1 ($P > 0.1$),

sions is not significantly different from linear, and its slope of 0.999 is not significantly different from one or from that of KCl. (The third curve labeled "frozen twice" will be discussed shortly.)

The plot for the suspensions was actually made in terms of the relative volume fraction of the suspension occupied by cells (a_v^{rel}) rather than the mass fraction of

water (a_w^{rel}). This was done because the differences in the estimates of a_v^{rel} from different methods were as great as the differences between a_v^{rel} and a_w^{rel} in a given method (*cf.* Tables I and II).

TABLE II

RELATIVE CELL CONCENTRATIONS (a_v^{rel}) AND MASS FRACTIONS OF WATER (a_w^{rel}) IN 1 ×, ½ ×, AND ¼ × YEAST SUSPENSIONS AS ESTIMATED BY SEVERAL TECHNIQUES

Concentration of cells	Mean hematocrit value	Cell count: Total (10^9/ml)	Cell count: Viable in control (10^9/ml)	Mean $a_v^{rel} \pm s_{\bar{x}}$	Calculated a_w^{rel}*
	per cent				
1 ×	41.5 (7)‡	3.70 (7)	3.04 (2)	1.00 ± 0.02	1
½ ×	21.5 (4)	1.94 (4)	1.37 (2)	0.512 ± 0.045	0.494
¼ ×	10.8 (4)	1.00 (4)	0.62 (2)	0.258 ± 0.015	0.245

* See last paragraph of Appendix A for method of calculation.
‡ Values in parentheses are numbers of observations.

2. The expected temperature differential (ΔT) between an ideal frozen solution and ice at any temperature (T_s) can be calculated by combining equations (9) and (10) to give

$$\Delta T = \frac{(\Delta T_{max})^2}{T_s} \tag{11}$$

One of the differential thermal plots of 1× cell suspensions, for example, yielded a value of 3.21°C for ΔT_{max}. In Fig. 11, this observed plot (ΔT *versus* T_s) is com-

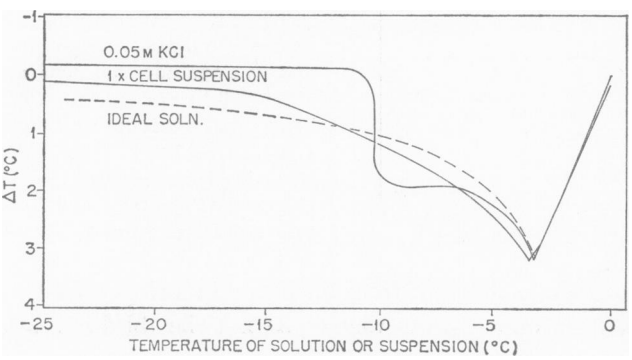

FIGURE 11 Observed differential thermal warming curve of a frozen 1× yeast suspension compared with that of frozen 0.05 M KCl and with the calculated curve for a theoretical solution with no eutectic point.

pared with that observed previously for frozen 0.05 M KCl and with that predicted for a theoretical frozen solution exhibiting the same ΔT_{max}; that is, the curve calculated from the equation

$$\Delta T = \frac{(3.21)^2}{T_s} = \frac{10.31}{T_s} \tag{12}$$

In making the calculation, the theoretical solution was assumed to have no eutectic point and, therefore, to melt progressively at all subzero temperatures.

In general, the plot for the cell suspension falls between the observed curve for KCl and the calculated curve. This intermediate position probably results from the fact that protoplasm, because of its variety of solutes, does not exhibit a unique eutectic point like KCl, but rather what Rey (1960) has referred to as "a eutectic zone"—a broad range of temperatures over which a number of eutectic points are exerting sequential and superimposed endothermic effects.

3. We shall see shortly that most of the osmotically active solutes in cells have leaked out after thawing. When a thawed 1× suspension was washed 5 times to remove these solutes, and the cells were resuspended in water and refrozen, the resulting value of $(\Delta T_{max})^2$ was reduced to 25 per cent of the original. Therefore, the amount of heat absorbed by suspensions during warming was determined by the concentration of solutes present and not by some unknown attributes of yeast cells.

4. The observed endothermic process could not have been due to heats of solution or dilution of dehydrated yeast solids. As shown in Table III, these heats are exothermic, not endothermic.

TABLE III

HEATS OF SOLUTION OF YEAST SOLIDS

Source of dried yeast	Weight added to 100 gm H_2O	Rise in H_2O temperature	Heat released per:	
			gm solids	0.15 gm solids*
	gm	°*C*	*cal*	*cal*
Commercial‡, 8 per cent water	2.00	0.054	2.7	0.40
Commercial‡, oven-dried	2.00	0.296	14.8	2.22
NRRL-Y-2235, freeze-dried	0.1566	0.025	16.0	2.40
	0.1158	0.017	14.7	2.21

* Weight of solids in 1 × cell suspension (see Appendix A, Table VIII).

‡ Fleishmann's active dry yeast.

Proportion of the Intracellular Solution Frozen by Rapid Cooling. To produce the observed quantity of heat, at least 80 per cent of the intracellular solution had to have been frozen and had to behave like a macroscopic frozen solution. This statement is based on the following data:

Solutes in medium before freezing. The heat absorbed by a warming suspension was much greater than that produced by the solutes present in the external water before freezing. With a 1× suspension, the heat was the same as that absorbed by frozen 0.05 M KCl (Fig. 10). With the external medium (isolated by centrifugation before freezing) it was only 1/25th as great because the medium contained a concentration of solutes equivalent to only 0.0020 to 0.0026 M KCl. These concentrations were estimated from the values of $(\Delta T_{max})^2$ and of melting point depressions obtained from DTA runs on the isolated suspending medium, and are given in Table IV.

TABLE IV

EFFECT OF REPETITIVE FREEZING AND THAWING ON THE THERMAL CHARACTERISTICS AND CONDUCTIVITY OF 1 × YEAST SUSPENSIONS

Treatment	Component	$(\Delta T_{max})^2$	Melting point lowering*	Conductivity
		$(°C)^2$	°C	*mho/cm*
Before freezing	Suspension	—	—	2.0×10^{-4}‡
	Suspending medium	0.44§	0.04§	2.0×10^{-4}
After 1st freezing	Suspension	10.0	0.56	35×10^{-4}
	Supernatant	9.8	—	45×10^{-4}
After 2nd freezing	Suspension	10.8	0.64	43×10^{-4}
After 3rd freezing	Suspension	11.2	—	—
After sonication and 4th freezing	Suspension	12.5	0.77	46×10^{-4}

* Melting points were obtained from DTA plots of ΔT *versus* time (t) like those in Fig. 8, and were taken as the temperature at which $d\,\Delta T/dt$ was minimum.

‡ Equivalent to the conductivity of 0.0014 M KCl. The conductivity of KCl is nearly a linear function of the molar concentration on the basis of data published by Robinson and Stokes (1959). The empirical relation is $\log L \approx -0.908 + 0.975 \log C_0$. The true relation is $\log L = \log (\Lambda/1000) + \log C_0$ where Λ is the molar conductance, a parameter varying somewhat with concentration.

§ The equivalent concentrations of KCl producing these values of $(\Delta T_{max})^2$ and melting point lowerings are 0.0020 and 0.0026 molal as calculated from the regression equations in Fig. 7.

Escape of solutes into medium after thawing. The next question is how the heat absorbed by a frozen 1× suspension (which equaled that absorbed by frozen 0.05 M KCl) compares to what would have been absorbed if all the intracellular solution had been frozen. This could be determined by converting the compartmented cell system to a true solution by disrupting the cell membranes, and a number of measurements showed that exactly this conversion occurred in the thawed suspensions (Table IV). The melting point depressions and electrical conductivities of 1× suspensions were 14 times or more higher during and after thawing, re-

spectively, than were those of the suspending media or suspensions before freezing; moreover, there was little difference between the conductivity of a thawed suspension and that of the suspending medium isolated after thawing, proving that intracellular electrolytes had indeed left the cells. When this isolated medium was itself frozen and subjected to DTA, it yielded the same shape warming curve and absorbed the same amount of heat $(\Delta T_{max})^2$ as that originally absorbed by the intact suspension. Thus the thawed suspension had not only been converted into a non-compartmented solution, but a solution that exhibited the same thermal characteristics as the original suspension.

Effect of multiple freezing and thawing. The only remaining question is whether a single cycle of freezing and thawing had converted all portions of all cells into true solutions. The question was investigated by subjecting the disrupted suspensions to additional cycles of freezing and thawing. The values of $(\Delta T_{max})^2$, conductivities, and melting point depressions, for twice-frozen 1× cells are shown in Table IV. The relation between these parameters and cell concentration are plotted in Figs. 10, 12, and 13, where they are compared with the values obtained for

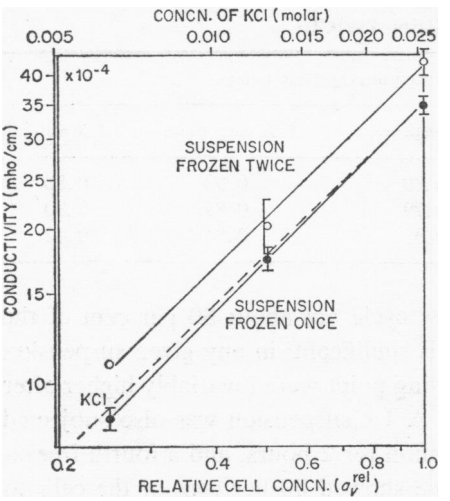

FIGURE 12 Electrical conductivities (L) of thawed yeast suspensions and of KCl as functions of the concentration of cells and of the solute concentration, respectively. The curve after the initial freezing of yeast is given by $\log L = -2.457 + 1.011 \log a_v^{rel}$, with the values of $s_{y.x}$, s_λ, and n being ±0.040, ±0.043, and 14, respectively. After the second freezing, the curve is $\log L = -2.381 + 0.982 \log a_v^{rel}$, with $s_{y.x}$, s_λ, and n being ±0.045, ±0.075, and 6. The curve for KCl is that for the equation given in the footnote (‡) of Table IV. None of the curves departs significantly from linear ($P > 0.5$), nor do their slopes differ significantly ($P > 0.7$).

suspensions frozen and thawed only once and with the values for KCl solutions.[2] The shapes of the DTA curves from which values of $(\Delta T_{max})^2$ were obtained were similar to those of singly frozen cells. From these figures and from Table V, it is

[2] The estimates of the concentrations of solutes (in terms of equivalent KCl values) from conductivity measurements were about half of those estimated from $(\Delta T_{max})^2$ and melting points. The latter two are colligative, but conductivity responds only to charged molecules. Furthermore, the numerical value of conductivity also depends on the equivalent or molar conductance of the specific ions. The major anions in yeast are amino acids (Conway and Armstrong, 1961), and they have a molar conductance less than half that of the Cl^- in the reference KCl solution (Schmidt, 1938; Robinson and Stokes, 1959).

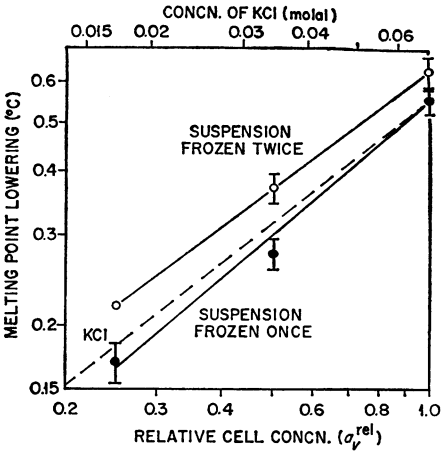

FIGURE 13 Melting point lowerings of cell suspensions compared with those of KCl solutions. The equation relating the lowering to cell concentration is log $T_m = -0.260 + 0.872 \log a_v^{rel}$ after the first freezing, with $s_{y.x}$, s_λ, and n being ±0.063, ±0.070, and 13, respectively. After the second freezing, the relation is log $T_m = -0.199 + 0.765 \log a_v^{rel}$ with $s_{y.x}$, s_λ, and n being ±0.039, ±0.058, and 7. The equation for KCl was given in Fig. 7. None of the curves departs significantly from linear ($P > 0.20$), and their slopes do not differ significantly ($P > 0.20$).

TABLE V

VALUES OF $(\Delta T_{max})^2$, MELTING POINTS, AND CONDUCTIVITY FOR SUSPENSIONS FROZEN ONCE COMPARED TO VALUES FOR SUSPENSIONS FROZEN TWICE

Parameter	Ratio: Frozen once/Frozen twice			
	1/4 × suspension	1/2 × suspension	1 × suspension	Mean
$(\Delta T_{max})^2$	0.68	0.80	0.93	0.80
Melting point	0.75	0.79	0.87	0.80
Conductivity	0.81	0.82	0.84	0.82

seen that the values after a single freeze-thaw cycle are about 80 per cent of the values after the second cycle. The difference is significant; in any given suspension the values of $(\Delta T_{max})^2$, conductivity, and melting point were invariably higher after the second freezing cycle than after the first. A 1× suspension was also subjected to three cycles of freezing and thawing, sonication for 2 hours, and a fourth freeze-thaw cycle. Examination under the microscope showed 80 per cent of the cells to be grossly broken or cracked; yet the values of our measures of solute concentration $(\Delta T_{max})^2$, melting point, and conductivity increased only slightly over the initial values (*cf.* Table IV).

If we assume that two cycles of freezing and thawing resulted in the complete conversion of the suspension into a solution, then we must conclude that about 80 per cent of the original intracellular solution was frozen after the initial freezing.

This assumption appears to be substantially correct, for it leads to values for the osmolal concentration of intracellular solutes that are comparable to those obtained by other investigators using other procedures. As shown in Table VI, the estimate from the thermal measurements is 0.54 osmolal for the twice-frozen cells. Eddy and

TABLE VI

ESTIMATED CONCENTRATION OF SOLUTES IN TWICE-FROZEN YEAST CELLS

Parameter	Concentration of cells	Value of parameter*	Equivalent solute concentration of suspension		a_w§	Osmolality of protoplast
			KCl‡	Osmolal		
$(\Delta T_{max})^2$, $(°C)^2$	1 ×	10.9	0.054	0.108	0.286	0.38
	½ ×	6.36	0.031	0.062	0.138	0.45
	¼ ×	3.71	0.018	0.036	0.068	0.53
Melting point lowering, °C	1 ×	0.63	0.083	0.166	0.286	0.58
	½ ×	0.37	0.043	0.085	0.138	0.62
	¼ ×	0.22	0.022	0.044	0.068	0.65
					Average	0.54

* From regression curves.
‡ Concentration of KCl giving same value of parameter, as calculated from the regression equations in Fig. 7.
§ *Cf.* Table I.

Williamson (1957) found that an isosmotic suspending solution for yeast protoplasts was 0.5 osmolar, and Conway and Armstrong (1961) have estimated the intracellular osmolarity of yeast to be 0.59 by cryoscopic measurements and by summing the colligative contributions of the known constituents of yeast protoplasm.

The conclusion that some 80 per cent of the water in the protoplasts was frozen agrees reasonably well with the elegant calorimetric determinations of frozen water by Wood and Rosenberg (1957) and by Souzu, Nei, and Bito (1961). Using the "method of mixtures" (Sturtevant, 1959), they determined that 90 per cent of the cell water was frozen at temperatures below −20°C. The residual unfrozen water probably was not supercooled but was water of low activity; *i.e.,* bound. The discrepancy between the estimates of 80 and 90 per cent could be due to differences in the strains of yeast used, but it is more likely due to the larger errors inherent in DTA or to the possibility that DTA of multiply frozen-thawed cells yielded too high an estimate of the solute concentration in normal intact cells. Nord (1933) has shown that multiple freezing and thawing can disaggregate the molecules in dilute colloidal solutions. If such a breakdown occurred in the twice-frozen yeast suspensions, the resulting increase in numbers of molecules would have inflated the estimate of solute concentration beyond the true value and, therefore, led to an underestimate of the percentage of water frozen in the initial cooling. The rough agreement between the calorimetric and DTA estimates of cellular water frozen shows that DTA provided a valid indication that the bulk of the protoplasm was frozen. But the unique contribution of DTA was to show that the frozen protoplasm behaved like a frozen solution, a point on which calorimetry is silent.

Membrane Inactness and Location of Solutes during Heat Absorption. We have seen that before freezing the solutes are inside the cell and separated from the external medium by an intact membrane and that, after thawing, they have diffused out of the cell and converted the suspension into a solution. To determine their location *before* thawing during the time when heat is absorbed, one may use the fact that electrolytes held within a cell by an intact membrane cannot contribute to the electrical conductivity of a suspension. Their inability to do so has been shown by Cole and many others (Cole and Curtis, 1950) to be a consequence of the shunting of low and moderate frequency measuring currents around a cell as a result of the high impedance of an intact plasma membrane. This condition prevailed before freezing, and a 1× suspension behaved like 0.0015 M KCl. It did not prevail after thawing, and the suspension then behaved like a 0.025 M KCl solution (Table IV and Fig. 12).

Suppose we compare the conductometric behavior of frozen 0.0015 M and 0.025 M KCl solutions during warming. Such a comparison is made in Fig. 14 where

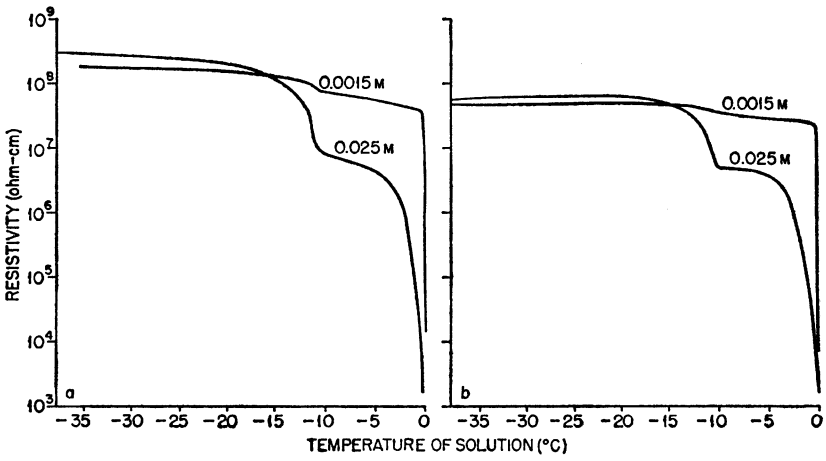

FIGURE 14 Changes in the electrical resistivity of frozen 0.0015 and 0.025 M KCl during warming: (*a*) Measurements at 250 CPS, (*b*) at 1000 CPS.

resistivity is plotted against temperature (resistivities were used so that changes would be in the same downward direction as the changes in ΔT in the DTA plots). The exact shape and position of the curves depended somewhat on the frequency of the measuring current,[3] but at a given frequency, the resistivity of the dilute 0.0015 M KCl underwent little change until the temperature had risen to nearly

[3] The plotted values of the resistances cannot be depended on as accurate measures of the absolute resistivity of ice and the frozen solutions. The bridge used did not have provisions for guarding. Consequently, the highest resistance values could well have been those of insulation leakage rather than that of the ice itself. However, here we are only concerned with the relative resistances of the different concentrations of KCl and cell suspensions at different temperatures.

0°C, at which point it dropped precipitously. In contrast, the resistivity of the more concentrated frozen 0.025 M KCl dropped slowly between −35° and −15°C, and then dropped suddenly between −15° and −10°C. With further warming, it continued to decrease, slowly at first, but then at an increasing rate as the temperature approached 0°C. The gradual initial drop had something to do with ions being present, for it did not occur with pure ice. The sudden drop between −15° and −10°C is near the eutectic point, and is undoubtedly a manifestation of initial melting (Rey, 1960). The decreases at higher temperatures reflect the progressive liquefaction of the solution.

Similar changes in resistance were noted during the warming of frozen 1× cell suspensions and are depicted in Fig. 15. Frozen-thawed suspensions subjected to a

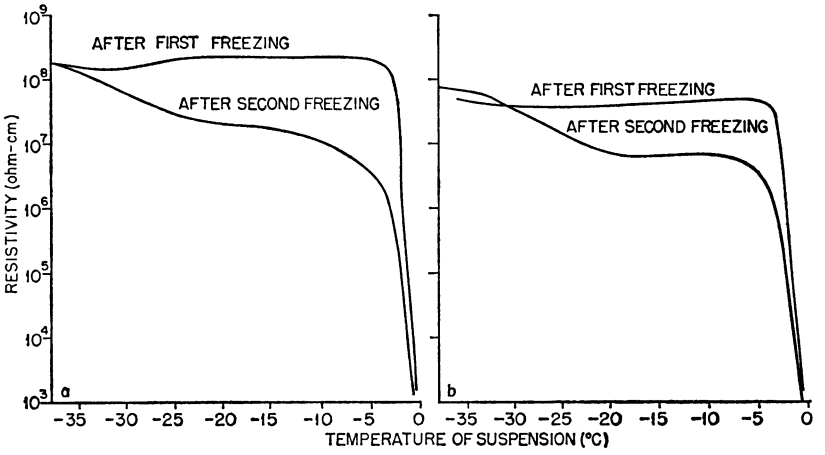

FIGURE 15 Changes in the electrical resistivity of once- and twice-frozen yeast suspensions during warming. (*a*) Measurements at 250 CPS, (*b*) at 1000 CPS.

second freezing changed resistance during rewarming in a manner similar to frozen 0.025 M KCl. This similarity was expected since the first cycle of freezing and thawing had destroyed permeability barriers and converted the suspension into a solution having the same conductivity as 0.025 M KCl. The chief difference between the two was the absence in the suspensions of an abrupt drop between −15° and −10°C, another indication that protoplasm lacks a defined eutectic point.

The changes in resistance during the warming of the 1× suspensions after the *initial* freezing were markedly different from the above and quite similar to the changes noted in the dilute 0.0015 M KCl solutions: The resistance remained high and constant until the temperature had risen to about −4°C, indicating that only the solutes originally present in the external medium before freezing were able to conduct current; since the great bulk of the solutes would not conduct, they must still have been inside the cell and surrounded by a membrane sufficiently intact to have prevented their outward diffusion. Reference to Figs. 9 and 11 will show that

heat absorption began at a much lower temperature than −4°C and reached its maximum ΔT by the time warming had progressed that far. Apparently then, the solutes responsible for the heat absorption were still compartmented in the cell at the time heat was being absorbed.

It might be thought that the resistivity of the initially frozen suspensions would remain high even if the cell membrane had been ruptured after freezing because the intracellular electrolytes would be constrained from diffusing by the surrounding ice. However, this argument is untenable. With the membrane ruptured, the intracellular solutes would be pockets of solute surrounded by and in direct contact with pure ice. But this is exactly the situation in any frozen solution such as KCl or the twice-frozen suspensions. As a solution freezes, pure ice separates out and the solutes are forced into channels between the ice crystals so that eventually the ice surrounds pockets of solute. We have seen that the resistivity in such a situation begins to drop as soon as the temperature rises above the eutectic point. Above that point, solutes begin to dissolve by melting the nearest ice. The solutes are then free to diffuse to fresh ice surfaces and to melt them. An ice lattice, therefore, would not, and did not, prevent solute diffusion. Solute diffusion would be prevented and resistivity would remain high only if the plasma membrane were sufficiently intact to prevent the outward passage of the solute molecules.

Above −4°C, the resistance of even these initially frozen cells dropped abruptly. The interpretation here is that disruption of membranes took place above −4°C, thus allowing the cell solutes to diffuse rapidly into the surrounding medium and contribute to the conductivity of the suspension.

DISCUSSION

The differential thermal measurements show that rapidly frozen suspensions of yeast cells obey the physical-chemical laws applicable to dilute solutions; their behavior is not affected measurably by biological or biochemical processes such as active transport. This being so, the four models discussed in the Introduction represent the four possibilities for the state of the cells at subzero temperatures.

Model I—Supercooled Cells. This possibility is eliminated by the differential thermal measurements. These show that 80 per cent or more of the intracellular water was frozen and not more than 20 per cent supercooled or bound. These values are comparable to those obtained from calorimetric measurements which yield figures of 90 and 10 per cent frozen and unfrozen water, respectively. The greater precision of the calorimetric technique, combined with the possibility already discussed that DTA resulted in an underestimate of the amount of cell water frozen, makes the latter set of percentages the more reliable.

Model II—Disruption of Membranes. The second alternative for yeast at subzero temperatures is that freezing causes membrane disruption and hence obliterates the distinction between "intracellular" and "extracellular." Although the

thermal behavior of the suspension is compatible with this model (which predicts behavior like a frozen solution), the model is ruled out by the resistance measurements on frozen suspensions. These measurements showed that the intracellular solutes were inside the cells and surrounded by membranes sufficiently intact to prevent their diffusing out of the cell at the time heat absorption was occurring.

Model III—Intracellular Ice Formation. The third model assumes that intracellular freezing occurs and converts the cells into compartments of frozen solution suspended in essentially pure ice. The thermal data are in accord with equations (9) and (10) and, therefore, with the model. Furthermore, the model assumes that the solutes are inside the cell. The resistance measurements indicated that this was the case.

Model IV—Dehydrated Cells. The fourth model assumes that sufficient water leaves the cells during cooling to prevent intracellular ice from forming at any temperature and, hence, assumes that the only water remaining in the cell is the 10 per cent that cannot freeze. Although this model is not distinguishable from the preceding on the basis of the quantity of heat absorbed during melting, it is theoretically distinguishable in that "melting" occurs by the diffusion of water or vapor across the cell membrane, a process requiring time; but diffusion, if it occurred, would probably be so rapid that no time lag would be detectable with the techniques used (Mazur, in press).

However, models III and IV differ in another particular; namely, they predict different effects of freezing on cell volumes. The resistance measurements showed that intracellular solutes did not leave the cell during cooling or during most of the subsequent warming. Therefore, any changes in the volumes of cells during cooling or warming would be equal to the volume of water leaving or entering (excluding minor effects of thermal contraction or expansion). Since the dehydration model demands that all freezable intracellular water leave the cells during cooling, it requires that the whole cell or the protoplast shrink by a volume equal to that of the 90 per cent of the water in a cell that is potentially freezable. Rapidly cooled cells do not shrink by that amount nor do they plasmolyze (Nei, 1960; Mazur, 1961*b;* Eddy, 1958). Nei observed no shrinkage in rapidly cooled yeast photographed on the freezing stage of a microscope. We observed that rapidly cooled yeast fixed at low temperatures by freeze-substitution with ethanol shrunk to 50 per cent of their original volume, indicating that about 66 per cent of the intracellular water had left the cell and 34 per cent remained inside (Table VII). To reduce the amount of intracellular water to the 10 per cent unfreezable level, the cells would have to have shrunk to one-third of their original volume. Since they did not do so, the dehydration model appears untenable in the freeze-substituted cells and is clearly untenable in the cells rapidly frozen by Nei. Slowly cooled cells on the other hand do shrink to one-third of their original volume and their volume is significantly less than that of those cooled rapidly (Mazur, 1961*b*).

TABLE VII

WATER CONTENT AS A FUNCTION OF CELL VOLUME

	Before freezing	After rapid cooling
Relative cell volume*	1	0.5
Volume of cell solids plus water in cell wall‡	0.24	0.24
Volume of intraprotoplast water	0.76	0.26
Per cent of intraprotoplast water still in cells	100	34

* Mazur, 1961*b*.

‡ Appendix A, Table VIII.

In conclusion, rapid cooling to −196°C followed by slow warming kills more than 99.999 per cent of cells of *Saccharomyces cerevisiae*. The conductometric behavior of the suspensions during warming indicates that the cells can be viewed as compartments of aqueous solution suspended in ice and separated from it by an intact cell membrane, and the thermal behavior indicates that, in the main, the intracellular solution obeys the thermodynamic laws applicable to bulk dilute solutions. One consequence of these facts is to permit the derivation of a quantitative kinetic description of water loss in cells at subzero temperatures and its relation to biological parameters, such as cell size and permeability to water as well as to the likelihood of internal freezing (Mazur, in press). As a result of rapid cooling, some 90 per cent of the intracellular water freezes.[4] Some of it leaves the cell to freeze externally, some of it freezes inside the cell; and the relative proportion in each location can be estimated from the volume of the cells before they are warmed if one knows what fraction of the cell was originally occupied by water. Knowing these proportions, and knowing that only 10 per cent of the water is unfreezable, one can determine whether internal ice formed in the cell and the fraction of the intracellular water that froze there. The two available estimates of the volumes of yeast after rapid cooling indicate that intracellular ice was present under these lethal conditions; however, quantitative estimates of the amount of ice must await more precise measurements of the volumes of frozen cells.

The cell membranes of rapidly frozen yeast are sufficiently intact after freezing and remain sufficiently intact during warming to prevent the loss of solutes until the temperature rises to about −4°C. At that temperature, permeability barriers break down and the intracellular solutes move rapidly out into the surrounding, partially melted medium.

[4] Neither the calorimetric measurements of Wood and Rosenberg (1957) and Souzu *et al.* (1961) nor the DTA measurements prove that this quantity necessarily had to be frozen at the end of cooling. It is conceivable that a portion vitrified during cooling and only completed crystallization during the early stages of slow warming.

APPENDIX A

WATER AND SOLIDS IN CELL SUSPENSIONS

A cell suspension of V_T ml consists of several regions: A fraction a_v is occupied by the cells, and $(1 - a_v)$ by extracellular water. The cells are assumed to consist of protoplasts surrounded by a cell wall. The fraction of the volume of the cell occupied by the total cell solids is α, that occupied by the solids in the cell wall is β, and that occupied by the water in the cell wall is γ. The volumes of all the regions of the suspension can be expressed in terms of these five parameters: a_v, V_T, α, β, and γ, and are so expressed in the first row of Table VIII. The remainder of Table VIII gives numerical values both to these volumes and to their masses.

Since the values of the five parameters were not known directly, they and the other numerical values had to be calculated from known quantities. These were the following:

1. The volume of suspensions (V_T) was 1.50 ml in all experiments.

2. The packed cells in 1× suspensions occupied 41.5 per cent of the volume of the suspension on the basis of hematocrit measurements. The fraction of the packed cells attributable to intercellular space was calculated to be 0.16 as follows: Conway and Downey (1950) obtained an interspace value of 0.23 for yeast centrifuged at 3000 *g;* and centrifugation at that acceleration yielded a hematocrit for our cells 1.083 times greater than the value of 0.415 obtained at 15,500 *g*. In other words, the cell pack volume decreased 8 per cent at the higher speeds, and assuming all this decrease to be due to a decrease in intercellular space, the latter had to be 0.16. Therefore a_v, the fractional volume occupied by the cells *per se*, was $0.415\ (1 - 0.16) = 0.35$. The fraction a_v was also calculated by multiplying the volume of cells + buds (calculated from micrometry) times the number of cells and buds in the suspension (hemocytometer counts). The mean number of cells and buds was 3.70×10^9/ml of suspension, and the mean cell and bud volume was 96 μ^3. The total cell volume was, therefore, 0.354 ml cells/ml suspension, and $a_v = 0.354$.

3. The density of the cell solids was taken to be 1.50 gm/ml. Pycnometric measurements on dried yeast solids yielded a density of 1.44 gm/ml. The higher value was chosen to make the values in Table VIII internally consistent.

4. A value of 1.10 gm/ml was used as the density for the whole cells. This is the mean of published values ranging from 1.06 to 1.14 gm/ml (Brace, 1950; Conway and Downey, 1950; Mazur, 1961c).

5. The concentration of solids in the suspension was 0.09 to 0.1 gm/gm of suspension on the basis of oven-drying. A figure of 0.1 gm/gm was used in the calculations.

6. A figure of 0.30 gm/gm was used for the mass fraction of the cell solids attributable to cell wall solids. This was based on an analysis by Falcone and Nickerson (1956).

7. The density of the cell wall solids was assumed to be 1.60 gm/ml (essentially that of carbohydrates).

8. Electron micrographs (Vitols, North, and Linnane, 1961; Hashimoto, Conti, and Naylor, 1959) indicate an average cell wall thickness of 0.1 μ; thus, the volume of the cell wall is approximately 10 per cent of the volume of the whole cell.

9. The specific heat of yeast cell solids was measured by Wood and Rosenberg (1957) as 0.25 cal/gm/°C. The value for ice is 0.5 cal/gm/°C.

From these nine values, the remaining values in the table can be computed, assuming that the volumes are additive: α, β, and γ become 0.196, 0.0554, and 0.0446, respectively.

TABLE VIII

FRACTIONS OF YEAST CELLS AND CELL SUSPENSIONS OCCUPIED BY SOLIDS AND BY WATER

	Protoplast solids	Cell wall solids	Total cell solids	Protoplast water	Cell wall water	Total cell water	Protoplast total	Cell wall total	Cell total	Extraprotoplast water	Extra-cellular water	Total water in suspension	Suspension total
Symbolically													
Volume	$(\alpha-\beta)a_vV_T$	βa_vV_T	αa_vV_T	$(1-\alpha-\gamma)a_vV_T$	γa_vV_T	$(1-\alpha)a_vV_T$	$(1-\beta-\gamma)a_vV_T$	$(\beta+\gamma)a_vV_T$	a_vV_T	$[1-a_v(1-\gamma)]V_T$	$(1-a_v)V_T$	$(1-\alpha a_v)V_T$	V_T
Relative values for cells ($a_vV_T = 1$)													
Volume, *ml*	0.141	0.055	0.196	0.760	0.044	0.804	0.900	0.100	1.000	—	—	—	—
Density, *gm/ml*	1.47	1.60	1.50	1.00	1.00	1.00	1.07	1.33	1.10	—	—	—	—
Mass, *gm*	0.208	0.089	0.295	0.760	0.044	0.804	0.966	0.133	1.100	—	—	—	—
1 × *cell suspensions* ($a_v = 0.35$, $V_T = 1.50$ ml)													
Volume, *ml*	0.074	0.029	0.103	0.399	0.023	0.422	0.472	0.052	0.525	0.998	0.975	1.397	1.50
Mass, *gm*	0.109	0.047	0.155	0.399	0.023	0.422	0.507	0.070	0.578	0.998	0.975	1.397	1.552
Heat capacity, *cal/°C* (as ice)	—	—	0.039	—	—	0.211	—	—	0.250	—	0.487	0.698	0.737
½ × *cell suspension* ($a_v = 0.175$, $V_T = 1.50$ ml)													
Volume, *ml*	0.037	0.015	0.051	0.200	0.012	0.211	0.236	0.026	0.262	1.249	1.238	1.448	1.50
Mass, *gm*	0.055	0.024	0.078	0.200	0.012	0.211	0.254	0.035	0.289	1.249	1.238	1.448	1.527
Heat capacity, *cal/°C* (as ice)	—	—	0.020	—	—	0.106	—	—	0.126	—	0.619	0.725	0.745
¼ × *cell suspension* ($a_v = 0.0875$, $V_T = 1.50$ ml)													
Volume, *ml*	0.018	0.007	0.026	0.100	0.006	0.106	0.118	0.013	0.131	1.375	1.369	1.474	1.50
Mass, *gm*	0.027	0.012	0.039	0.100	0.006	0.106	0.127	0.017	0.144	1.375	1.369	1.474	1.513
Heat capacity, *cal/°C* (as ice)	—	—	0.010	—	—	0.053	—	—	0.063	—	0.684	0.737	0.747

Values of a_w were calculated from the relations

$$a_w = \frac{(1 - \alpha - \gamma)a_s}{(1 - \alpha a_s)} \quad \text{and}$$

$$a_w^{\text{rel}} = \frac{a_{w_1}}{a_{w_2}} = \frac{a_{s_1}}{a_{s_2}}\left(\frac{1 - \alpha a_{s_2}}{1 - \alpha a_{s_1}}\right) = a_s^{\text{rel}}\left(\frac{1 - \alpha a_{s_2}}{1 - \alpha a_{s_1}}\right)$$

APPENDIX B

Standard errors for the mean values for ΔT_{max}, $(\Delta T_{max})^2$, melting point depressions, and conductivities of cell suspensions and KCl solutions are summarized in Table IX. Statistics for the regression curves were given in the figure legends.

TABLE IX

STANDARD ERRORS AND PERCENTAGE ERRORS OF ΔT_{max}, $(\Delta T_{max})^2$, MELTING POINT DEPRESSIONS, AND CONDUCTIVITIES OF FROZEN CELL SUSPENSIONS AND KCl SOLUTIONS

		Standard error ($s_{\bar{x}}$)			Percentage error ($s_{\bar{x}}/\bar{x}$)		
		1/4 × cells	1/2 × cells	1 × cells	1/4 × cells	1/2 × cells	1 × cells
Parameter	Units	0.01 M KCl	0.05 M KCl	0.1 M KCl	0.01 M KCl	0.05 M KCl	0.1 M KCl
					per cent	*per cent*	*per cent*
ΔT_{max}							
Cells frozen once	°C	0.13(4)*	0.17(4)	0.07(9)	8.3	7.1	2.2
Cells frozen twice	°C	0.13(2)	0.30(2)	0.16(4)	6.9	11.3	4.9
KCl	°C	0.04(6)	0.05(4)	0.07(3)	2.8	1.5	1.5
$(\Delta T_{max})^2$							
Cells frozen once	$(°C)^2$	0.44(4)	0.77(4)	0.42(9)	17.8	13.2	4.2
Cells frozen twice	$(°C)^2$	0.50(2)	1.61(2)	1.06(4)	14.0	22.7	9.8
KCl	$(°C)^2$	0.12(6)	0.32(4)	0.59(3)	5.7	3.0	3.0
Melting points							
Cells frozen once	°C	0.016(3)	0.019(3)	0.029(7)	9.3	6.8	5.1
Cells frozen twice	°C	0 (2)	0.026(2)	0.045(3)	0	7.0	7.2
KCl	°C	0.021(6)	0.016(6)	0.023(3)	16.7	3.7	3.2
Conductivities							
Cells frozen once	*mho/cm*	0.38(4)	0.88(4)	1.36(6)	4.4	5.0	3.9
Cells frozen twice	*mho/cm*	0.06(2)	2.53(2)	2.49(2)	0.6	12.4	5.9

*Numbers in parentheses are the number of replicates.

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REFERENCES

BRACE, K. C., Effects of X-rays on size of yeast cells. *Proc. Soc. Exp. Biol. and Med.*, 1950, **74**, 751.

COLE, K. S., and CURTIS, H. J., Bioelectricity: electric physiology, *in* Medical Physics (O. Glasser, editor), Chicago, Year Book Publishers, Inc., 1950, **2**, 82.

CONWAY, E. J., and ARMSTRONG, W. McD., The total intracellular concentration of solutes in yeast and other plant cells and the distensibility of the plant-cell wall, *Biochem. J.*, 1961, **81**, 631.

CONWAY, E. J., and DOWNEY, M., An outer metabolic region of the yeast cell, *Biochem. J.*, 1950, **47**, 347.

DANIELS, F., MATHEWS, J. H., WILLIAMS, J. W., BENDER, P., and ALBERTY, R. A., Experimental Physical Chemistry, New York, McGraw Hill Book Co., 5th edition, 1956, 43.

EDDY, A. A., Aspects of the chemical composition of yeast, *in* The Chemistry and Biology of Yeasts (A. H. Cook, editor), New York, Academic Press, Inc., 1958, 229.

EDDY, A. A., and WILLIAMSON, D. H., A method of isolating protoplasts from yeast, *Nature*, 1957, **179**, 1252.

FALCONE, G., and NICKERSON, W. J., Cell-wall mannan-protein of Baker's yeast, *Science*, 1956, **124**, 272.

GLASSTONE, S., Textbook of Physical Chemistry, New York, D. Van Nostrand Co., 2nd edition, 1946, 643, 747

HASHIMOTO, T., CONTI, S. F., and NAYLOR, H. B., Studies of the fine structure of microorganisms. IV. Observations on budding *Saccharomyces cerevisiae* by light and electron microscopy, *J. Bact.*, 1959, **77**, 344.

International Critical Tables (E. W. Washburn, editor), New York, McGraw-Hill Book Co., 1928, **4**, 259.

MAZUR, P., Physical factors implicated in the death of microorganisms at subzero temperatures, *Ann. N. Y. Acad. Sc.*, 1960, **85**, 610.

MAZUR, P., Physical and temporal factors involved in the death of yeast at subzero temperatures, *Biophysic. J.*, 1961*a*, **1**, 247.

MAZUR, P., Manifestations of injury in yeast cells exposed to subzero temperatures. I. Morphological changes in freeze-substituted and in "frozen-thawed" cells, *J. Bact.*, 1961*b*, **82**, 662.

MAZUR, P., Manifestations of injury in yeast cells exposed to subzero temperatures. II. Changes in specific gravity and in the concentration and quantity of cell solids, *J. Bact.*, 1961*c*, **82**, 673.

MAZUR, P., Kinetics of water loss from cells at subzero temperatures and the likelihood of intracellular freezing, *J. Gen. Physiol.*, data to be published.

NEI, T., Effects of freezing and freeze-drying on micro-organisms, *in* Recent Research in Freezing and Drying, (A. S. Parkes and Audrey U. Smith, editors), Oxford, England, Blackwell Scientific Publications, 1960, 78.

NORD, F. F., Die Kryolyse und ihre Beziehung zum Mechanismus der Enzymwirkung, *in* Ergebnisse der Enzymforschung, (F. F. Nord and R. Weidenhagen, editors), Leipzig, Akademische Verlagsgesellschaft M. B. H., 1933, **2**, 23.

REY, L. R., Thermal analysis of eutectics in freezing solutions, *Ann. N. Y. Acad. Sc.*, 1960, **85**, 510.

ROBINSON, R. A., and STOKES, R. H., Electrolyte Solutions, New York, Academic Press, Inc., 1959, 465.

SCHMIDT, C. L. A., Electrochemistry of amino acids and proteins, *in* The Chemistry of the Amino Acids and Proteins (C. L. A. Schmidt, editor), Springfield, Illinois, Charles C. Thomas, Publishers, 1938.

SEIDELL, A., Solubilities of Inorganic and Metal Organic Compounds, New York, D. Van Nostrand Co., Inc., 3rd edition, 1940, **1**, 747.

SKAU, E. L., The purification and physical properties of organic compounds. I. The interpretation of time-temperature curves in freezing point determinations and as a criterion of purity, *Proc. Am. Acad. Arts and Sciences,* 1933, **67**, 551.

SMITH, A. U., Biological Effects of Freezing and Supercooling, Monograph No. 9 of the Physiological Society, Baltimore, Williams and Wilkins Co., 1961.

SMOTHERS, W. J., and CHIANG, Y., Differential Thermal Analysis, New York, Chemical Publishing Co., 1958.

SOUZU, H., NEI, T., and BITO, M., Water of microorganisms and its freezing, *Low Temp. Sc., Series B.,* 1961, **19**, 49.

STURTEVANT, J. M., Calorimetry, *in* Physical Methods of Organic Chemistry, 3rd edition, *Technique of Organic Chemistry* (A. Weissberger, editor), New York, Interscience Publishers, Inc., 1959, **1**, pt. 1.

VITOLS, E., NORTH, R. J., and LINNANE, A. W., Studies on the oxidative metabolism of *Saccharomyces cerevisiae.* I. Observations on the fine structure of the yeast cell, *J. Biophysic. and Biochem. Cytol.,* 1961, **9**, 689.

WOOD, T. H., and ROSENBERG, A. M., Freezing in yeast cells, *Biochim. et Biophysica Acta,* 1957, **25**, 78.